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Short communication

Neospora caninum antibodies detected in Midwestern white-tailed deer (*Odocoileus virginianus*) by Western blot and ELISA

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Abstract

White-tailed deer (*Odocoileus virginianus*) serve to maintain the *Neospora caninum* life cycle in the wild. Sera from white-tailed deer from south central Wisconsin and southeastern Missouri, USA were tested for antibodies to *N. caninum* by Western blot analyses and two indirect ELISAs. Seroreactivity against *N. caninum* surface antigens was observed in 30 of 147 (20%) of WI deer and 11 of 23 (48%) of MO deer using Western blot analysis. Compared to Western blot, the two indirect ELISAs were found to be uninformative due to degradation of the field-collected samples. The results indicate the existence of *N. caninum* antibodies in MO and WI deer, and that Western blot is superior to ELISA for serologic testing when using degraded blood samples collected from deer carcasses.

Keywords: *Neospora caninum*; White-tailed deer (*Odocoileus virginianus*); Seropositivity; Western blot; ELISA; Missouri; Wisconsin

1. Introduction

Neosporosis caused by the apicomplexan parasite *Neospora caninum* is a major cause of bovine abortion worldwide (Dubey, 2003). The life cycle involves canids as definitive hosts and ruminants as intermediate hosts. Sylvatic transmission occurs between canids (such as coyotes) and ruminants (such as white-tailed deer) (Gondim et al., 2004a,b; Vianna et al., 2005). We are currently investigating the Midwestern sylvatic cycle, including the possibility that other mammals can act as hosts for *N. caninum*. Here we report *N. caninum* seropositivity in white-tailed deer (*Odocoileus virginianus*) from two Midwestern sites (WI and MO) based on Western blot analyses, and the evaluation of two indirect enzyme-linked immunosorbent assays (ELISAs) for detection of circulating anti-*Neospora* antibodies in hunted deer.

2. Materials and methods

Whole blood samples were collected from the thoracic cavities of 147 hunter killed white-tailed deer at the Wisconsin Department of Natural Resource hunter check station in Sauk City, WI (November 2004, 2005), and from 23 deer at a Missouri Department of Natural Resource hunter check station in Ste. Genevieve County, MO (November 2001). Samples were separated by centrifugation immediately after collection and the serum stored at −20 °C. Two *Neospora* agglutination

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test (NAT, Romand et al., 1998) positive and two NAT negative white-tailed deer (Dubey et al., 1999) were used as controls. WB analysis was used to confirm the presence or absence of circulating antibodies against *N. caninum*. A defined WB protocol (Howe et al., 1998) was modified by using 1:1000-diluted rabbit anti-deer antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as a secondary reagent. Samples were considered seropositive if antibody binding was observed for the Ncp29 (SAG1) and Ncp 35 (SRS2) antigens (Howe et al., 1998). Seropositivity was calculated by dividing the number of seropositive deer by the total number of deer screened and multiplied by 100.

A Ncp29 cattle ELISA (Howe et al., 2002) was modified for use with deer serum by using horseradish peroxidase-conjugated rabbit anti-deer IgG. The assay was optimized by varying the following conditions: (a) test of bovine serum albumin (BSA), nonfat dry milk, fetal bovine serum and rabbit serum in coating and antibody diluent solutions, (b) temperature at which assay was conducted (room temperature versus 37 °C), (c) phosphate buffered saline [PBS] versus polyethylene glycol blocking solutions, and (d) type of microtiter plate used (Immulon 4, Thermo Electron, Milford, MA; EIA/RIA Corning, Corning, NY). The assay was conducted at room temperature using Corning plates blocked with BSA in PBS, deer serum and horseradish peroxidase-conjugated rabbit anti-deer IgG at 1:1000, and washing plates four times prior to addition of chromogenic *O*-phenylenediamine. Absorbance at 492 nm (A_{492}) was determined using a SPECTRAmax Plus 384 ELISA plate reader (Molecular Devices, Sunnyvale, CA). All samples were tested in triplicate. Absorbance values were normalized by subtraction of the mean absorbance of two wells that had been treated in the absence of primary antibody.

An indirect ELISA using whole *N. caninum* tachyzoite lysate was developed as an alternative to the recombinant antigen ELISA. Whole cell-culture derived NC-1 strain tachyzoites (Dubey et al., 1988) were boiled for 5 min in 1× non-reducing Laemmli buffer (0.100 mM Tris pH 6.8, 4% SDS, 10 % glycerol). Protein concentration was estimated using the bicinchoninic acid protein assay (Sigma Chemical, St. Louis, MO). Microtiter plate wells were coated with 10 μg/mL tachyzoite lysate in PBS for 4 °C overnight (Howe et al., 2002). The remainder of the assay was conducted as for the optimized recombinant antigen assay, except that the assay was evaluated at 4 and 37 °C. Missouri deer samples were tested in triplicate, Wisconsin samples in duplicate. For both ELISAs, the percent positivity of each sample was calculated using the following equation: PP = [(mean sample A_{492} − mean negative control A_{492})/(mean positive control A_{492} − mean negative control A_{492})] × 100 (Wright et al., 1993). The sensitivity and specificity of each assay were calculated with PP cutoff values of 0, 10 and 20.

3. Results

Western blot analysis revealed seroreactivity against immunodominant *N. caninum* surface antigens in 30 of 147 (20%) of WI deer and 11 of 23 (48%) of MO deer (Fig. 1). Twenty-five WI samples (4 of which were WB positive) and 23 MO samples (11 of which were WB positive) were selected to assess the performance of the NcP29 ELISA. Mean negative control A_{492} values ranged from 0.212 to 0.468, while A_{492} values for positive controls ranged from 0.680 to 2.491. Percent positivity of WB-negative test samples ranged from 27.8 to 631.2, and from 23.6 to 183.1 for Western blot positives (Fig. 2A). The highest combined sensitivity (91%) and specificity (70%) was attained using a positive cutoff of 0 PP, which was deemed unacceptable for a high throughput screening tool. To determine if the presence of EDTA could improve the accuracy of the assay, additional samples were collected in EDTA vacuum tubes. However the resulting plasma was completely uninformative in the recombinant antigen ELISA (data not shown).

In an effort to design an assay with higher specificity that could be used for screening numerous samples, we developed an alternate ELISA using whole parasite

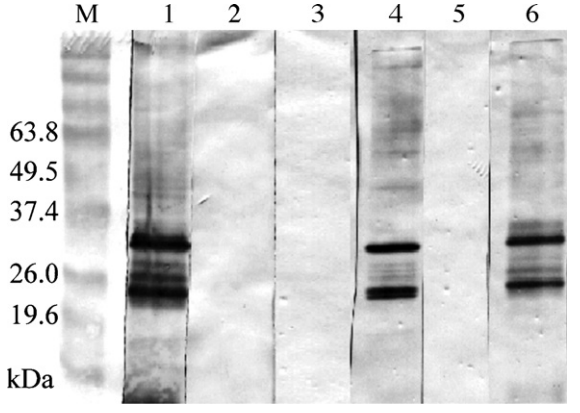

Fig. 1. Western blot analysis of deer serums samples using rabbit anti-deer secondary antibody. Immunodominant proteins of 35 and 25 kDa were bound by the agglutination positive deer (lane1), WI (lane 4), and MO (lane 6) deer; but not by the agglutination negative deer (lane 2), WI (lane 3), and MO (lane 5) samples. Molecular weights were estimated by comparison to Kaleidoscope prestained standard (M) (Bio-Rad, Hercules, CA).

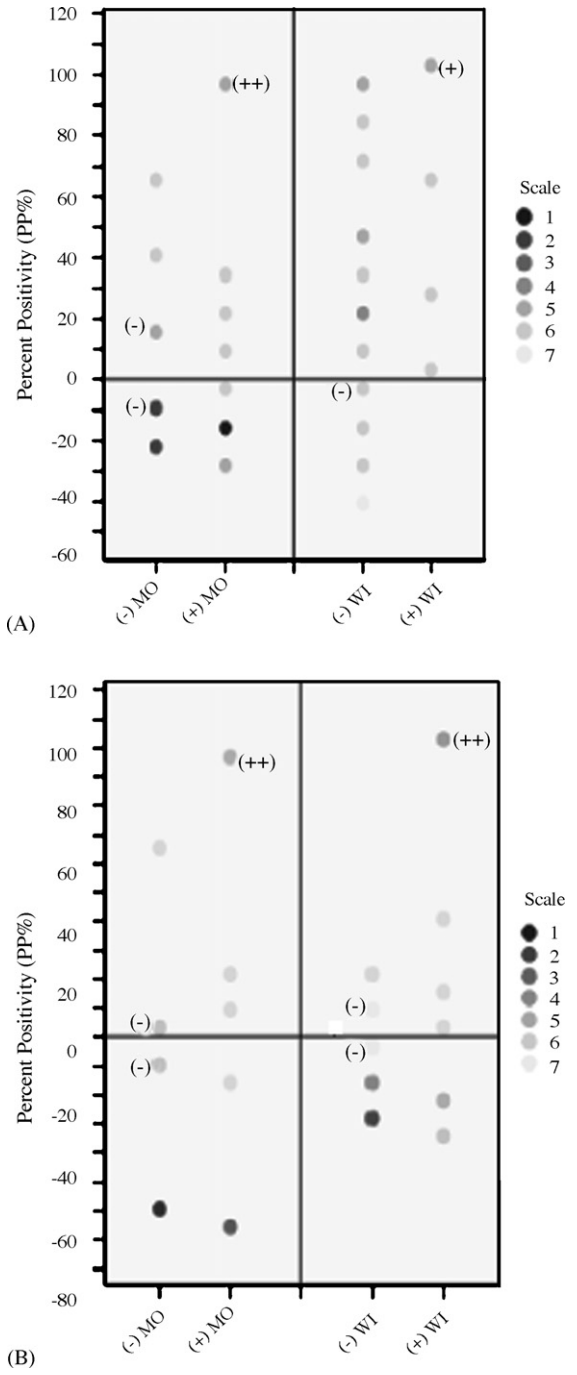

Fig. 2. Performance of indirect ELISAs with field-collected serum samples. Panel A: recombinant antigen (Ncp29) assay. Panel B: whole tachyzoite antigen assay. Values are expressed as percent positivity, the number of samples in each data point is indicated by the shade scale. Shown are test samples from Western blot negative (−MO, −WI) and positive (+MO, +WI) deer from Missouri and Wisconsin, as well as NAT-positive and negative control samples from Illinois (indicated in chart by (+, −) symbols). The presence of ++ indicates that two positive control samples overlap on one data point.

lysate as the target antigen. Forty-two WI samples (10 of which were WB positive) and the 23 MO samples were selected to test the validity of the assay. The groups were tested separately, with Wisconsin samples run in duplicate, MO in triplicate. Negative control values ranged from 0.131 to 0.245, and positive control values ranged from 0.340 to 1.023. Percent positivity of WB-negative test samples ranged from −65.45 to 26.4, while WB positive test values ranged from 65.5 and 169.0 (Fig. 2B). The assay performed with high sensitivity (up to 91% at a cutoff of 0 PP), however the specificity of the assay ranged between 52 and 71%. The performance of the assay also differed between sample groups, with higher sensitivity and lower specificity observed in the WI samples (data not shown). To determine if serum samples had degraded due to poor collection conditions, the total IgG concentration of the WI and MO samples were examined. Binding of anti-deer secondary antibody to serum dilutions indicated that the total IgG concentration in the control sera was at least two orders of magnitude greater than the MO and WI samples (data not shown).

4. Discussion

The overall purpose of our research is to obtain evidence of the sylvatic *N. caninum* life cycle in central Wisconsin, with which to qualify future studies on transmission in regional wildlife species. Importantly, our data demonstrated that WB is a much better technique for screening deer serum samples because it is more informative and less sensitive to sample degradation than ELISA. Seropositivity of 20 and 48% was observed in the two Midwestern sites, which agrees with previous report of approximately 40% in neighboring Illinois deer (Dubey et al., 1999). Mounting evidence points to a role of white-tailed deer as natural intermediate hosts in the sylvatic life cycle of *N. caninum* (Gondim et al., 2004a; Lindsay et al., 2002; Vianna et al., 2005) and to linkage of the domestic and sylvatic cycles in some areas (Tiemann et al., 2005). It is possible that management programs aimed at prevention and control of neosporosis may need to factor in situations in which cattle have extensive contact with wildlife.

N. caninum tissue cysts are not histologically apparent in adult cattle and, presumably, deer; hence serological methods such as direct agglutination test (Packham et al., 1998), indirect fluorescent antibody test (Conrad et al., 1993), ELISA (for review see Dubey and Schares, 2006), immunoblot (Bjerkas et al., 1994),

and rapid immunochromatographic test (Liao et al., 2005) are undertaken to screen populations for exposure. Evaluation of serological results from wildlife serum samples is often difficult because of the degradation of antibodies after death. This is particularly more applicable to serologic tests that use color as an indicator, e.g. the ELISA tests. In contrast to ELISA, Western blot analysis gives a direct visual confirmation of antibody bound to specific diagnostic antigens (i.e., NcP29 and NcP35), thereby providing greater confidence in the results. Although the NAT test is simple and can be used with autolysed samples, NAT is no longer available commercially (J.P. Dubey, personal information). Therefore, we evaluated ELISA and WB in the present study.

Collection of deer blood samples for biological research is often performed at hunter check stations, from animals that have been eviscerated in the field. Many factors contribute to the degradation of serum proteins, such as length of time since death of the animal, ambient temperature in the field, presence or absence of ice in the body cavity, contamination of the cavity with debris, and sample storage conditions. It is difficult to collect high quality blood samples in this situation, and the researcher must be prepared to evaluate them using an assay that performs well under these circumstances (Greiner and Gardner, 2000). In this case, WB was superior to ELISA for establishing evidence of *N. caninum* exposure in Midwestern deer.

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References

Bjerkas, I., Jenkins, M.C., Dubey, J.P., 1994. Identification and characterization of *Neospora caninum* tachyzoite antigens useful for diagnosis of neosporosis. Clin. Diagn. Lab. Immunol. 1, 214–221.

Conrad, P.A., Sverlow, K., Anderson, M., Rowe, J., BonDurant, R., Tuter, G., Breitmeyer, R., Palmer, C., Thurmond, M., Ardans, A., et al., 1993. Detection of serum antibody responses in cattle with natural or experimental *Neospora infections*. J. Vet. Diagn. Invest. 5, 572–578.

Dubey, J., Hollis, K., Romand, S., Thulliez, P., Kwok, O., Hungerford, L., Anchor, C., Etter, D., 1999. High prevalence of antibodies to *Neospora caninum* in white-tailed deer (*Odocoileus virginianus*). Int. J. Parasitol. 29, 1709–1711.

Dubey, J.P., 2003. Neosporosis in cattle. J. Parasitol. 89 (Suppl.), S42–S46.

Dubey, J.P., Hattel, A.L., Lindsay, D.S., Topper, M.J., 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J. Am. Vet. Med. Assoc. 193, 1259–1263.

Dubey, J.P., Schares, G., 2006. Diagnosis of bovine neosporosis. Vet. Parasitol. 140, 1–34.

Gondim, L.F., McAllister, M.M., Mateus-Pinilla, N.E., Pitt, W.C., Mech, L.D., Nelson, M.E., 2004a. Transmission of *Neospora caninum* between wild and domestic animals. J. Parasitol. 90, 1361–1365.

Gondim, L.F., McAllister, M.M., Pitt, W.C., Zemlicka, D.E., 2004b. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 34, 159–161.

Greiner, M., Gardner, I.A., 2000. Application of diagnostic tests in veterinary epidemiologic studies. Prev. Vet. Med. 45, 43–59.

Howe, D., Tang, K., Conrad, P., Sverlow, K., Dubey, J., Sibley, L., 2002. Sensitive and specific identification of *Neospora caninum* infection of cattle based on detection of serum antibodies to recombinant Ncp29. Clin. Diagn. Lab. Immunol. 9, 611–615.

Howe, D.K., Crawford, A.C., Lindsay, D., Sibley, L.D., 1998. The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of *Toxoplasma gondii*. Infect. Immun. 66, 5322–5328.

Liao, M., Zhang, S., Xuan, X., Zhang, G., Huang, X., Igarashi, I., Fujisaki, K., 2005. Development of rapid immunochromatographic test with recombinant NcSAG1 for detection of antibodies to *Neospora caninum* in cattle. Clin. Diagn. Lab. Immunol. 12, 885–887.

Lindsay, D.S., Little, S.E., Davidson, W.R., 2002. Prevalence of antibodies to *Neospora caninum* in white-tailed deer, *Odocoileus virginianus*, from the southeastern United States. J. Parasitol. 88, 415–417.

Packham, A.E., Sverlow, K.W., Conrad, P.A., Loomis, E.F., Rowe, J.D., Anderson, M.L., Marsh, A.E., Cray, C., Barr, B.C., 1998. A modified agglutination test for *Neospora caninum*: development, optimization, and comparison to the indirect fluorescent-antibody test and enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. 5, 467–473.

Romand, S., Thulliez, P., Dubey, J.P., 1998. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitol. Res. 84, 50–53.

Tiemann, J.C., Souza, S.L., Rodrigues, A.A., Duarte, J.M., Gennari, S.M., 2005. Environmental effect on the occurrence of anti-*Neospora caninum* antibodies in pampas-deer (*Ozotoceros bezoarticus*). Vet. Parasitol. 134, 73–76.

Vianna, M.C., Sreekumar, C., Miska, K.B., Hill, D.E., Dubey, J.P., 2005. Isolation of *Neospora caninum* from naturally infected white-tailed deer (*Odocoileus virginianus*). Vet. Parasitol. 129, 253–257.

Wright, P.F., Nilsson, E., Van Rooij, E.M., Lelenta, M., Jeggo, M.H., 1993. Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Rev. Sci. Tech. 12, 435–450.